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14. ABSTRACT
The recurrence of breast cancer (BrCa) at distal, metastatic sites, such as in the lung or in the bones, presents the greatest impact on patient mortality. We propose to identify genes that promote or suppress metastatic growth of BrCa, and in doing so, identify potential genetic signatures useful in either predicting or diagnosing BrCa spread or as potential new targets for anti-BrCa drugs. The metastasis-suppressor (MetSup) or -inducer (MetInd) genes were identified by introducing two different genomic screening virus pools, GIPZ or VBIM, into tagged human BrCa cells (T47D/luciferase) with poor metastatic growth potential, and then selecting for cells that gain high metastatic potential in a cell culture assay. Preliminary analysis has begun on analyzing these potential inducers (MetInd) or suppressors (MetSup) of BrCa metastasis by analyzing human BrCa tissue arrays.

15. SUBJECT TERMS
Breast cancer, metastasis, invasiveness, shRNA, RNAi, genomic libraries, BT474, T47D, retrovirus, lentivirus, Matrigel.

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Introduction:

We proposed to use a novel and revolutionary technology based on so-called RNA-interference (RNAi), in which specific genes can be turned off using tailored RNAi molecules. The field of RNAi is only a decade old, and the PI is the Director of a CORE Facility at RPCI that houses one of the first RNAi libraries covering all known human genes (~35,000 genes). Thus, we proposed to turn off individual genes in a population of poorly-invasive human BrCa cells by treating them with an entire human genomic RNAi library. In contrast, we also infected them with a VBIM retrovirus that can induce occult gene expression, thereby also inducing increased invasiveness. We selected for cells that have significantly increased metastatic behavior using two techniques: i) multiple rounds of Matrigel invasion assays in Boyden chambers to select for clones with increasing invasive potential, and ii) sealing the infected cells into collagen plugs, and then implanting these into fibrinogen gels, followed by isolation of cells that invaded into the surrounding fibrinogen. The cells with increased invasive potential were cloned, subjected to bar-code-specific PCR and sequencing to identify novel metastasis-regulating genes. In several cases, follow-up analyses with different siRNA reagents were used to validate that the knockdown of specific genes altered invasiveness without altering proliferation. Finally, we attempted to determine whether the up- or downregulation of specific genes correlated with metastasis or advanced BrCa using Oncomine or GEO. The identity of these genes will help in understanding the signaling pathways required for cancer malignancy, and in the case of the potential MetInd genes, they may be potential therapeutic targets to prevent or treat BrCa metastasis.

Body:

Below are each of the tasks approved in the Statement of Work followed by a report on how the tasks aims were met (or not), or how experiments were adapted to address the intent of the tasks (in bold)

Task 1. Transduce BrCa cells with shRNA library and VBIM: Infect and select BT474/luciferase cells with Decode® GIPZ human genomic shRNA lentivirus library (selection: GFP-positive, puromycin-resistant) or VBIM retrovirus (selection: G418-resistant). Produce negative control (BT474/luciferase infected with empty GIPZ lentivirus) and positive control (BT474/ luciferase transduced with TWIST1 cDNA) cell lines.

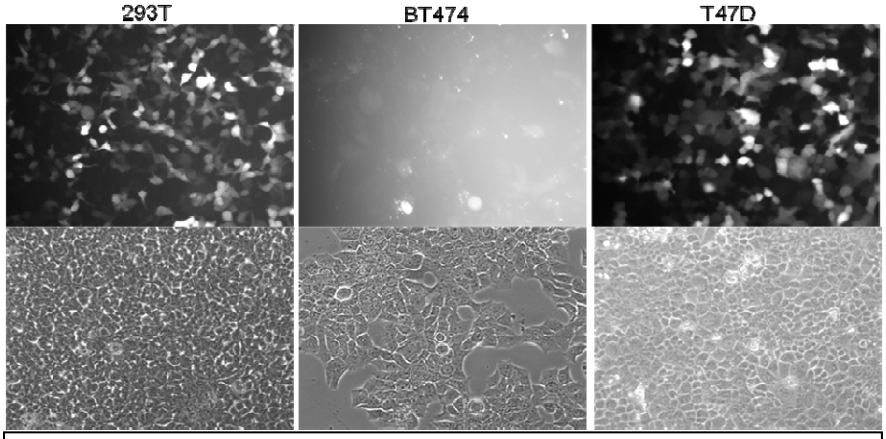

Fig. 1. Equal aliquots of high-titer pGIPZ virus (empty vector; expressing IRES-GFP) was used to infect HEK293T, BT474 or T47D cells. The number of GFP-positive cells (top row) was determined after 2 days versus total cells (bottom; phase contrast).

Initial tests on the BT474 cells (parental and /luciferase clones) showed poor infectivity with control pGIPZ lentivirus (empty vector) based on GFP-positive cells. Thus, after testing a panel of human BrCa cells with relatively low Matrigel invasive potential, we settled on T47D cells, which are highly infectable by pGIPZ (Fig. 1). A version was then produced that was transduced with luciferase. These cells were infected with 7 pools of pGIPZ-shRNA (10,000 clones/aliquot) lentivirus or control (empty pGIPZ), or with VBIM retrovirus, and then selected for the appropriate drug marker (puroR or G418R). Roughly 50,000/Matrigel-coated Boyden chamber were plated, with 8-10 chambers/virus aliquot pool. A positive control for increased invasiveness was T47D/luciferase cells transduced with TWIST1 cDNA (not shown).

Task 2. Select for variants with increased Matrigel invasiveness: Subject the shRNA- and VBIM-transduced BT474/luciferase cells to at least 3 rounds of Matrigel invasion assays. Isolate the invasive cells after each round by removing the cells atop the Matrigel layers, and then isolating the cells under the transwell membrane after trypsinization. Expand these cultures, and then repeat the Matrigel invasion assay. The shRNA- and VBIM-infected cells should show progressively increasing levels of invasiveness over vector alone controls.

Cells with increased Matrigel invasiveness were selected by at least three rounds (Fig. 2; "series 1-3" = rounds of Matrigel selection) of Boyden chamber assays, with increases in rounds 2-4 in shRNA aliquots #3, #6 and #7, and with the VBIM, compared to control viruses.

Task 3. Identify MetSup and MetInd genes: Produce clones of the highly invasive BT474/luciferase-shRNA or –VBIM variants. For the shRNA clones, perform "bar-code"-specific PCR, sequence the PCR products, identify the knocked down genes using the Open Biosystems website (www.openbiosystems.com). Demonstrate gene knockdown by either Q-PCR or immunoblot (IB) using RNA or protein from the shRNA clones vs. empty vector controls. For the VBIM clones, isolate variant cell genomic DNA, cut with Not I enzyme, perform inverse PCR using VBIM-specific primer sets, sequence the PCR products. Verify that the VBIM-induced genes are expressed in the invasive cell variants compared to vector control cells by Q-PCR or immunoblot.

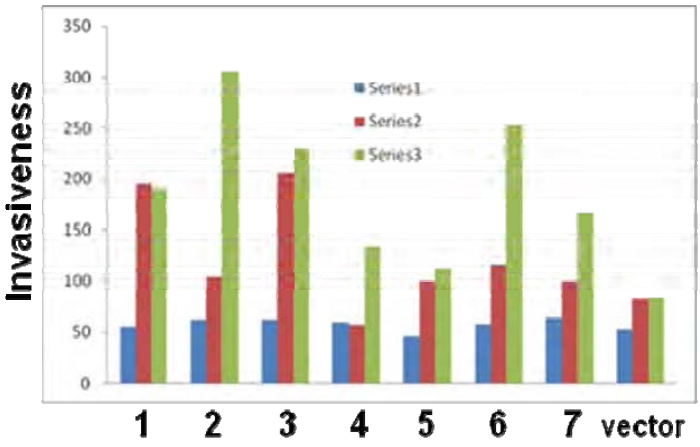

Fig. 2. Increased invasiveness of shRNA-expressing T47D BrCa cells after 3 rounds (series) of Matrigel invasion assays (compared to empty pGIPZ vector).

For each round 4 aliquot with increased Matrigel invasiveness, 3-10 cm plates containing 100 cells were plated and then after 1.5 weeks, 20 colonies in each plate were picked and expanded. Using PCR primers flanking the shRNA insert, 550 bp fragments were amplified and then sequenced. The most interesting genes included members of the FOXO family of transcription factors, Kinesin family, and SUMO-regulating genes. Commercial antibodies were obtained and IB analyses showed knockdown of the specific targeted protein. In several cases, T47D/luciferase cells were transfected with gene-specific siRNA pools (Dharmacon or IDT), and after demonstrating gene knockdown by IB, we also showed that loss of these genes increased Matrigel invasiveness.

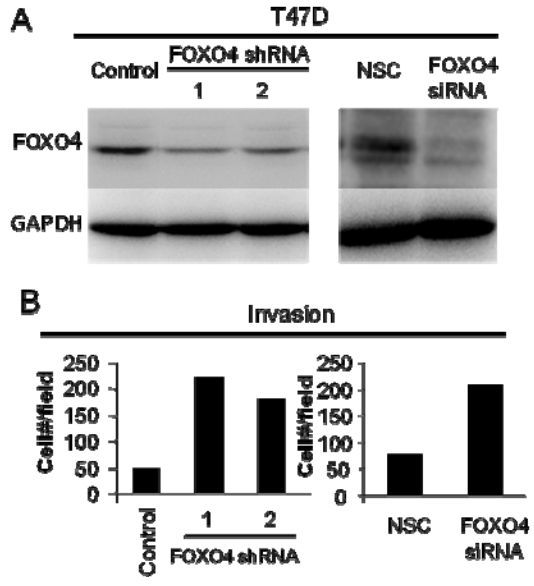

Fig. 3. (A) FOXO4 Immunoblot of FOXO4 shRNA or siRNA vs. control treated BrCa cells, showing protein knockdown compared to the control GAPDH protein. (B) Loss of FOXO4 results in >4-fold increase in Matrigel invasiveness.

We verified that the knockdown of several of these genes (FOXO4, KIF3B and SENP1) using gene-specific siRNA could increase the invasive potential of T47D cells (see Fig. 3 for FOXO4 data). Importantly, we found no increase in apoptosis or decrease in proliferation in the cells with stable MetSup knockdown (data not shown). We are surveying a panel of BrCa cell lines with varying metastatic/invasive potentials to

determine whether there is an inverse correlation between the expression levels of our identified MetSup genes and invasive potential.

We are also producing stable BrCa cell lines (e.g.- MDA-MB-231 or -435) with high invasive potential with forced re-expression of the MetSup genes, and these will be tested for decreased levels of Matrigel invasiveness, as well as changes to apoptotic and proliferative potentials. Lastly, we are also testing whether other family members can compensate for the loss of the MetSup activity. For, example, T47D cells also express high levels of FOXO1 and FOXO3a, yet only knockdown of FOXO4 induces increased metastatic potential.

Task 4. Compare validated MetSup and MetInd genes with BrCa microarray data in Oncomine and GEO: Identify microarray data sets that compare primary vs. metastatic (especially bone metastases) human BrCa patient and/or cell lines. Determine which MetSup or MetInd gene expression profiles track with BrCa metastasis.

We analyzed both Oncomine and GEO data for potential changes in human metastases vs. primary tumors or normal tissue. Fig. 4A shows a statistically significant decrease in mean FOXO4 RNA level in breast metastases (bone and lymph node) compared to primary-site DCIS or normal breast (GEO Study: GDS3139). Fig. 4B shows similar results, namely a small but significant drop in FOXO4 levels in metastases compared to primary lesions (Bittner Breast study). Taken together, these suggest that increased metastatic potential in human breast cancer correlates with lower expression levels of FOXO4.

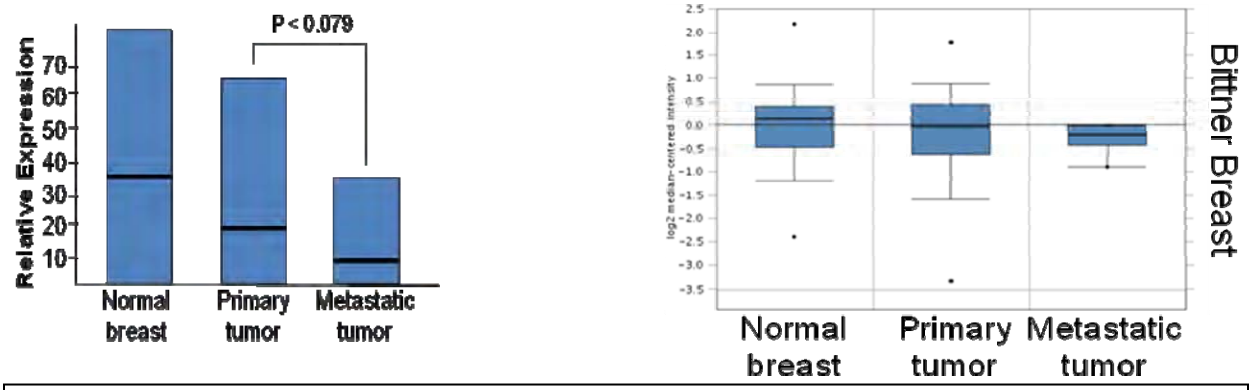

Fig. 4. (A; left) GEO Record GDS3139: FOXO4 mRNA levels in BrCa. (B; right) Oncomine- Bittner Breast Cancer comparison of FOXO4 mRNA levels.

Task 5- Validate MetSup or MetInd expression changes in human BrCa TMAs: Using no more than 3 MetSup and 3 MetInd genes validated in Task 4, human BrCa TMAs containing matched normal, primary and metastasis tissues will be probed by ISH or IHC. If appropriate staining Abs can be obtained from commercial or researcher sources (must identify single cognate protein bands in SDS-PAGE analysis), then the TMAs will be analyzed by IHC after the Abs have been tested for appropriate staining titers. If such Abs are not available, the TMAs will be analyzed by ISH using anti-sense RNA probes. cDNA samples will be obtained (Origene) from which labeled anti-sense RNAs will be produced (Ambion kits) and then used to hybridize to the TMAs, with labeled sense RNA probes as controls. Staining signals will be digitized and quantified by Aperio Scanning, followed by statistical correlation to metastasis formation and outcome data such as survival (using de-identified information).

We have not yet started this application, however, we are presently requesting breast cancer TMAs that are being produced by the RPCI Pathology Resource Network that will include, importantly, metastatic lesions (usually lymph node metastases).

Task 6- Test for MetSup and MetInd gene activity *in vivo*: BT474/luciferase cells will be transduced with one candidate MetInd gene or with one MetSup shRNA from the validation analysis in Task 5. The MetInd/MetSup expression l BT474/luciferase evels will be verified by Q-PCR or immunoblot. Female SCID mice (10/group) will be injected with 10^6 BT474/luciferase[MetInd], BT474/luciferase[MetSup], BT474/luciferase[vector], or BT474/luciferase[TWIST] cells, and bone OL growth will be monitored weekly for 2 months by i.v. luciferin injection (tail vein) followed one hour later by Xenogen BioImaging, or by Faxitron X-Ray imaging.

In progress using the T47D/uciferase[vector] or [FOXO4-shRNA] cells.

Task 7. Analysis and writing of report. **Done.**

Key Research Accomplishments:

- successfully transduced T47D BrCa cells with luciferase and then shRNAs, using our cancer gene-specific retrovirus shRNA sub-library and our GIPZ lentivirus human genomic library.
- successful selection (puromycin resistance for the retrovirus clones, GFP expression for the GIPZ clones) and expansion of these BrCa cells *in vitro*.
- demonstration that the shRNA-infected (vs. empty vector) cells exhibited greatly enhanced invasive potentials over control cells.
- isolation of individual shRNA-expressing BrCa clones for bar-code-specific PCR identification of knocked down genes.
- Confirm gene-specific knockdown by immunoblot of protein products; confirm using siRNA to same gene.
- Demonstrate that knockdown of identified gene induces increased Matrigel invasiveness in T47D without affecting cell survival or proliferation.
- Confirmed several novel MetSup genes based on their loss in metastatic BrCa based on GEO and Oncomine results
- Obtained cDNA expression vectors for the identified genes and family members; produced stable re-expressing BrCa cell lines that start with high invasive potentials (MDA-MB-231 and -435).
- established pools of shRNA-infected BrCa cells to be used in orthotopic injections of nude mice.

Reportable Outcomes:

- isolation and validation of a cancer-specific retrovirus shRNA library and a human genomic shRNA library in lentiviruses (GIPZ).
- isolation of T47D cell pools with stably transduced genomic shRNAs.
- isolation of T47D cells with increased spontaneous Matrigel invasiveness, and with even greater invasive potential after shRNA transduction.
- isolation of individual shRNA-infected and spontaneous T47D clones exhibiting enhanced invasiveness.

Conclusion:

We have successfully produced human BrCa clones that are stably transduced with shRNAs clones representing the entire human genome. We also isolated BrCa clones with increased spontaneous and shRNA-induced invasive potentials using *in vitro* selection systems. We are in the process of identifying and validating the individual genes whose knockdown potentially increases BrCa invasiveness *in vitro* and *in vivo*. Given that our data indicate greatly increased invasive potentials in the shRNA-infected clones over controls, there is confidence that these transduced pools will also yield increased rates of metastatic colonization and growth using orthotopic BrCa models in immunodeficient mice.

References:
none

Appendices:
none